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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
25 July 2002 (25.07.2002)

PCT

(10) International Publication Number  
**WO 02/057294 A2**

- (51) International Patent Classification<sup>7</sup>: C07K 94115 (US). LI, Guofu [CN/US]; 634 Stannage Avenue, Apt. B, Albany, CA 94706 (US).
- (21) International Application Number: PCT/US02/01906
- (22) International Filing Date: 22 January 2002 (22.01.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
60/263,445 22 January 2001 (22.01.2001) US  
60/290,716 11 May 2001 (11.05.2001) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:  
US 60/263,445 (CIP)  
Filed on 22 January 2001 (22.01.2001)  
US 60/290,716 (CIP)  
Filed on 11 May 2001 (11.05.2001)
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- (81) Designated States (*national*): AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 02/057294 A2

(54) Title: ZINC FINGER PROTEINS FOR DNA BINDING AND GENE REGULATION IN PLANTS

(57) Abstract: Disclosed herein are modified plant zinc finger proteins; compositions comprising modified plant zinc finger proteins and methods of making and using modified plant zinc finger proteins. The modified plant zinc finger proteins, in contrast to naturally-occurring plant zinc finger proteins, have a binding specificity that is determined by tandem arrays of modular zinc finger binding units.

## ZINC FINGER PROTEINS FOR DNA BINDING AND GENE REGULATION IN PLANTS

### TECHNICAL FIELD

The methods and compositions disclosed herein relate generally to the field of regulation of gene expression and specifically to methods of modulating gene expression in plants by utilizing polypeptides derived from plant zinc finger-nucleotide binding proteins.

### BACKGROUND

Zinc finger proteins (ZFPs) are proteins that bind to DNA, RNA and/or protein, in a sequence-specific manner, by virtue of a metal stabilized domain known as a zinc finger. See, for example, Miller *et al.* (1985) *EMBO J.* 4:1609-1614; Rhodes *et al.* (1993) *Sci. Amer.* Feb:56-65; and Klug (1999) *J. Mol. Biol.* 293:215-218. There are at least 2 classes of ZFPs which co-ordinate zinc to form a compact DNA-binding domain. Each class can be distinguished by the identities of the conserved metal-binding amino acids and by the associated architecture of the DNA-binding domain.

The most widely represented class of ZFPs, known as the C<sub>2</sub>H<sub>2</sub> ZFPs, comprises proteins that are composed of zinc fingers that contain two conserved cysteine residues and two conserved histidine residues. Over 10,000 C<sub>2</sub>H<sub>2</sub> zinc fingers have been identified in several thousand known or putative transcription factors. Each C<sub>2</sub>H<sub>2</sub> zinc finger domain comprises a conserved sequence of approximately 30 amino acids that contains the invariant cysteines and histidines in the following arrangement: -Cys-(X)<sub>2-4</sub>-Cys-(X)<sub>12</sub>-His-(X)<sub>3-5</sub>-His (SEQ ID NO: 1). In animal genomes, polynucleotide sequences encoding this conserved amino acid sequence motif are usually found as a series of tandem duplications, leading to the formation of multi-finger domains within a particular transcription factor.

Several structural studies have demonstrated that the conserved C<sub>2</sub>H<sub>2</sub> amino acid motif folds into a beta turn (containing the two invariant cysteine residues) and an alpha

helix (containing the two invariant histidine residues). The alpha helix and beta turn associate along a hydrophobic interface and are held together through the tetrahedral coordination of a zinc atom by the conserved cysteines and histidines.

The three-dimensional structure of a complex between a DNA target site and a polypeptide comprising three C<sub>2</sub>H<sub>2</sub> zinc fingers derived from the mouse immediate early protein zif268 (also known as Krox-24) has been determined by x-ray crystallography. Pavletich *et al.* (1991) *Science* 252:809-817. The structure reveals that the amino acid side chains on each zinc finger alpha helix interact specifically with functional groups of the nucleotide bases exposed in the DNA major groove. Each finger interacts with DNA as a module; changes in the sequence of amino acids of the recognition helix can result in corresponding changes in target site specificity. See, for example, Wolfe *et al.* (1999) *Annu. Rev. Biophys. Biomol. Struct.* 3:183-212.

Another class of ZFPs includes the so-called C<sub>3</sub>H ZFPs. See, *e.g.*, Jiang *et al.* (1996) *J. Biol. Chem.* 271:10723-10730 for a discussion of Cys-Cys-His-Cys (C<sub>3</sub>H) ZFPs.

The modular nature of sequence-specific interactions between zinc fingers and DNA sequences (*i.e.*, a particular zinc finger of defined sequence binds to a DNA triplet or quadruplet of defined sequence) allows certain DNA-binding domains of predetermined specificity to be designed and/or selected. See, for example, Blackburn (2000) *Curr. Opin. Struct. Biol.* 10:399-400; Segal *et al.* (2000) *Curr. Opin. Chem. Biol.* 4:34-39. To this end, numerous modifications of animal C<sub>2</sub>H<sub>2</sub> zinc finger proteins, most often either mouse zif268 or human SP-1, have been reported. See, *e.g.*, U.S. Patent Nos. 6,007,988; 6,013,453; 6,140,081; 6,140,466; GB Patent No. 2,348,424; PCT WO98/53057; PCT WO98/53058; PCT WO98/53059; PCT WO98/53060; PCT WO98/54311; PCT WO00/23464; PCT WO 00/42219; Choo *et al.* (2000) *Curr. Opin. Struct. Biol.* 10:411-416; Segal *et al.* (2000) *supra*; and references cited in these publications. The results of these and other studies are generally consistent with the idea that it is possible to obtain C<sub>2</sub>H<sub>2</sub> ZFPs, based on, for example, the mouse zif268 ZFP or the human SP-1 ZFP, of desired target site specificity. Such target-specific ZFPs are generally obtained by selection or design of individual fingers, each of which has a 3-4

nucleotide target specificity, and assembly of such fingers into a ZFP having a target site specificity of 9-20 nucleotides.

C<sub>2</sub>H<sub>2</sub> ZFPs have been identified in plants, where they are involved in, for example, developmental regulation of various floral and vegetative organs. See, e.g., Takatsuji (1999) *Plant Mol. Biol.* 39:1073-1078. In plant ZFPs, however, zinc fingers do not generally occur in closely-spaced tandem arrays. For example, in a family of DNA binding proteins identified in *Petunia* (the EPF family), two canonical Cys<sub>2</sub>-His<sub>2</sub> zinc finger motifs are separated by an intervening stretch of between 19 and 232 amino acids. The binding capability of this class of proteins appears to be determined by both the zinc fingers and the intervening amino acids, suggesting that plant zinc finger proteins have a different mechanism of DNA binding than do the zif268 and SP-1 zinc finger proteins, for example. In addition, the sequence specificity of DNA binding by EPF-type plant ZFPs is dependent upon different positions in the recognition helix of the zinc finger than is the specificity of DNA binding by most zif268-type ZFPs. See, for example, Takatsuji (1996) *Biochem. Biophys. Res. Comm.* 224:219-223.

Targeted gene regulation in plants would facilitate numerous applications such as, for example, the optimization of crop traits affecting nutritional value, yield, stress tolerance, pathogen resistance, and resistance to agrochemicals. In addition, targeted gene regulation could be used to study gene function in plants, and to adapt plants for use as biological factories for the production of pharmaceutical compounds or industrial chemicals. Such regulation could theoretically be achieved by design of plant transcriptional regulatory proteins of predetermined DNA sequence specificity. However, to date, naturally occurring plant ZFPs that recognize DNA by using a tandem arrangement of modular zinc finger binding domains (as do zif268 and related ZFPs) have not been described. Therefore, it remains difficult, if not impossible, to design a plant ZFP capable of recognizing and binding to a particular predetermined nucleotide sequence. Furthermore, since the mechanism of DNA binding by plant ZFPs remains largely unknown, no immediate solution to this problem is apparent. Accordingly, the ability to design and/or select plant zinc finger proteins of predetermined target specificity would be desirable.

### SUMMARY

The present disclosure provides plant DNA-binding proteins that are modified in such a way that their mechanism and specificity of DNA binding are determined by tandem arrays of modular zinc finger binding units. In this way, design strategies and selection methods which have been developed and utilized for other classes of ZFPs can be applied to the production of plant ZFPs having a predetermined target site specificity, for use in modulation of gene expression in plant cells.

In one aspect, disclosed herein is a modified plant zinc finger protein (ZFP) that binds to a target sequence. The target sequence can be, for example, nucleic acid (DNA or RNA) or amino acids of any length, for instance 3 or more contiguous nucleotides. In certain embodiments, the modified plant ZFP comprises a tandem array of zinc fingers. One, more than one or all of the zinc fingers of the ZFP may be naturally occurring or may be obtained by rational design and/or selection (*e.g.*, phage display, interaction trap, ribosome display and RNA-peptide fusion). Thus, in certain embodiments, one or more of the zinc fingers comprise canonical C<sub>2</sub>H<sub>2</sub> zinc fingers and in other embodiments, one or more of the zinc fingers comprise non-canonical zinc fingers. In any of the modified plant ZFPs described herein, one or more of the zinc fingers are derived from two or more plant species, for example, by deleting and/or substituting one or more amino acid residues as compared to a naturally occurring plant ZFP. In certain embodiments, one or more amino acid residues are deleted between one or more of the zinc fingers.

Thus, in one embodiment, plant zinc finger proteins (ZFPs) are modified, for example, by deletion of inter-zinc finger sequences and/or insertion of additional zinc finger sequences, to generate one or more tandem arrays of zinc fingers. Thus, in contrast to naturally occurring plant zinc finger proteins, their mechanism and specificity of DNA binding are determined by tandem arrays of modular zinc finger binding units. In another embodiment, plant zinc fingers of disparate origin (*e.g.*, zinc fingers from *Petunia* and *Arabidopsis*) are recombined into a tandem array of modular zinc finger binding units.

In yet another aspect, a fusion polypeptide comprising (i) a modified plant ZFP as described herein and (ii) at least one functional domain are described. The functional domain may be a repressive domain or an activation domain.

In yet another aspect, isolated polynucleotides encoding any of the modified plant zinc finger proteins or fusion polypeptides described herein are provided. Also provided are expression vectors comprising these polynucleotides. Also described are host cell comprising these polynucleotides and/or expression vectors.

In another aspect, a method for modulating gene expression in a plant cell comprising contacting the cell with any of the modified plant ZFPs described herein is provided. In one embodiment, the protein comprising a tandem array of zinc fingers is provided. Preferably, the protein is expressed in the cell, for example, by introducing the protein and/or a nucleic acid encoding the protein into the cell. In certain embodiments, the zinc fingers of the protein comprise an adapted amino acid sequence at any one or more of residues -1 through +6 of the recognition helix. The adapted amino acid sequence can be obtained by rational design and/or by selection (*e.g.*, using methods such as phage display, interaction trap, ribosome display, RNA-peptide fusion or combinations of one or more of these methods). In certain embodiments, the protein comprises zinc finger backbones from different species, for example different plant species. In other embodiments, the protein comprises zinc finger backbones of plant origin, fungal origin or combinations thereof. Furthermore, in certain embodiments, the protein is obtained by deletion of inter-finger sequences from a plant ZFP.

In other aspects, the methods described herein make use of a fusion protein comprising a tandem array of zinc fingers and one or more functional domains, for example, one or more transcriptional activation (*e.g.*, C1, etc.) or repression domains.

In other aspects, the compositions and methods described herein find use in a variety of applications in which modulation of gene expression alters the phenotype and/or composition of the plant or plant cell, for example by optimizing crop traits such as nutritional value, yield, stress tolerance, pathogen resistance, resistance to agrochemicals (*e.g.*, insecticides and/or herbicides) and the like; and by adapting plants for use in production of pharmaceutical compounds and/or industrial chemicals. In certain embodiments, the modulation of gene expression can be used to study genetic pathways and/or gene functions in plants.

These and other embodiments will readily occur to those of skill in the art in light of the disclosure herein.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic depicting construction of the YCF3 expression vector useful in expressing modified plant ZFPs.

Figure 2 shows the results of analysis of GMT mRNA in RNA isolated from *Arabidopsis thaliana* protoplasts that had been transfected with constructs encoding fusion of a transcriptional activation domain with various modified plant ZFPs. Results are expressed as GMT mRNA normalized to 18S rRNA. AGMT numbers on the abscissa refer to the modified plant ZFP binding domains shown in Table 2. Duplicate TaqMan<sup>®</sup> analyses are shown for each RNA sample.

### DETAILED DESCRIPTION

#### **General**

The present disclosure provides modified plant ZFPs (and functional fragments thereof), wherein zinc fingers are arranged in one or more tandem arrays such that, upon DNA binding, each zinc finger contacts a triplet or quadruplet target subsite. In preferred embodiments, the target subsites are contiguous to one another. The modified plant ZFP can be a fusion polypeptide and, either by itself or as part of such a fusion, can enhance or suppress expression of a gene (*i.e.*, modulate gene expression). Polynucleotides encoding modified plant ZFPs, and polynucleotides encoding fusion proteins comprising one or more modified plant ZFPs are also provided. Additionally provided are compositions comprising, in combination with an acceptable carrier, any of the modified plant zinc finger binding polypeptides described herein or functional fragments thereof; and compositions comprising a nucleotide sequence that encodes a modified plant zinc finger binding polypeptide or functional fragment thereof, wherein the modified plant zinc finger polypeptide or functional fragment thereof binds to a cellular nucleotide sequence to modulate the function of the cellular nucleotide sequence.

Currently, ZFPs targeted to specific predetermined sequences are derived from non-plant ZFPs such as *Xenopus* TFIIIA, murine *zif268*, human SP-1 and the like. Accordingly, in one embodiment, modified plant zinc finger proteins, targeted to predetermined sequences, are described wherein all or substantially all of the sequences



making up the ZFP are derived from one or more plant sources. Furthermore, the modified plant ZFPs are organized in non-plant ZFP structures, for example structures in which individual zinc fingers (*e.g.*,  $C_2H_2$  fingers) are linked by short linker sequences, or structures that do not contain native plant DNA binding sequences such as inter-zinc finger sequences of a plant zinc finger protein, (which can be generated from plant ZFPs, for example, by deletion of inter-zinc finger amino acid sequences). In certain embodiments, all amino acid residues of a modified plant ZFP are derived from a non-modified plant ZFP (*e.g.*, when a modified plant ZFP is obtained by deletion of inter-finger sequences from a non-modified plant ZFP). In other embodiments, one or more amino acid residues of a modified plant ZFP (*e.g.*, amino acids involved in sequence-specific and/or non-specific DNA contacts) can be either designed or selected, and thus may not constitute part of the original plant ZFP sequence.

It is preferred that a modified plant zinc finger protein be a multi-finger protein, for example comprising at least three zinc-coordinating fingers. In the standard nomenclature for ZFPs, the "first" finger is the N-terminal-most finger of the protein (with respect to the other fingers) and binds to the 3'-most triplet (or quadruplet) subsite in the target site. Additional fingers, moving towards the C-terminus of the protein, are numbered sequentially.

In other embodiments, one or more of the component fingers of the modified plant ZFP will be a non- $C_2H_2$  structure. For example, in certain embodiments, a three-finger zinc finger protein is provided wherein the first two fingers are of the  $C_2H_2$  class but the third finger is non- $C_2H_2$  (*e.g.*,  $C_3H$  or other structure) as described, for example, in International Publication entitled "Modified Zinc Finger Proteins" filed even date herewith, Attorney docket No. 8325-0025.40).

Therefore, the modified plant ZFPs disclosed herein differ from previously described designed zinc finger protein transcription factors in that they are entirely or primarily composed of plant sequences. Nonetheless, the plant sequences are assembled such that the overall structure of the binding region of the modified plant protein is similar to that of a non-plant eukaryotic zinc finger. Thus, modified plant ZFPs, as disclosed herein, comprise plant sequences either for the entire ZFP or for most of the ZFP. In the latter case, plant sequences are used preferably in all regions except those residues involved in recognition and/or binding

to the target site, which can comprise, for example, sequences obtained by rational design and/or selection.

It will be readily apparent that various combinations of zinc fingers can be used in a single modified plant ZFP. For example, all of the finger components can be designed (*i.e.*, their sequences are obtained as a result of rational design methods); all of the finger components can be selected (*i.e.*, their sequences are obtained by a selection method such as, *e.g.* phage display, two-hybrid systems or interaction trap assays); all of the finger components can be naturally-occurring plant zinc fingers; or the component fingers of a modified plant ZFP can be any combination of naturally-occurring plant zinc fingers, designed fingers and selected fingers.

In additional embodiments, the modified plant zinc finger proteins described herein (and/or functional fragments thereof) are used in fusion proteins, for example fusions of a modified plant ZFP DNA-binding domain with, *e.g.*, a repression domain, an activation domain, a chromatin remodeling domain, a component of a chromatin remodeling complex, a methyl-binding domain, a methyltransferase, an insulator-binding protein, and/or functional fragments thereof. Polynucleotides encoding any of the zinc finger proteins, components thereof, functional fragments thereof, and fusions thereof are also provided.

In additional embodiments, methods for modulating gene expression in plant cells, using modified plant ZFPs are provided. Because naturally-occurring plant ZFPs, which modulate plant gene expression *in vivo*, do not contain zinc fingers in tandem arrays, the ability of a ZFP containing a tandem array of zinc fingers to modulate gene expression in a plant cell is a surprising discovery. Thus, the compositions and methods disclosed herein allow the insights gained from work with non-plant ZFPs such as *zif268* and Sp-1 to be applied to regulation of plant gene expression by plant proteins; so that targeted regulation of gene expression in plant cells can be achieved by mechanisms similar to those already described for animal cells. In addition, by allowing targeted regulation of plant gene expression by plant proteins, the present methods and compositions will help to allay potential concerns regarding the introduction of animal proteins into plants.

The practice of the disclosed methods employs, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, genetics, computational

chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. See, for example, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989; Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; and the series METHODS IN ENZYMOLOGY, Academic Press, San Diego.

### Definitions

The terms "nucleic acid," "polynucleotide," and "oligonucleotide" are used interchangeably and refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogues of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties. In general, an analogue of a particular nucleotide has the same base-pairing specificity; *i.e.*, an analogue of A will base-pair with T.

The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. The term also applies to amino acid polymers in which one or more amino acids are chemical analogues or modified derivatives of a corresponding naturally occurring amino acid, for example selenocysteine (Bock *et al.* (1991) *Trends Biochem. Sci.* 16:463-467; Nasim *et al.* (2000) *J. Biol. Chem.* 275:14,846-14,852) and the like.

A "binding protein" is a protein that is able to bind non-covalently to another molecule. A binding protein can bind to, for example, a DNA molecule (a DNA-binding protein), an RNA molecule (an RNA-binding protein) and/or a protein molecule (a protein-binding protein). In the case of a protein-binding protein, it can bind to itself (to form homodimers, homotrimers, *etc.*) and/or it can bind to one or more molecules of a different protein or proteins. A binding protein can have more than one type of binding activity. For example, zinc finger proteins have DNA-binding, RNA-binding and protein-binding activity. A "binding profile" refers to a plurality of target sequences that are recognized and bound by a particular binding protein. For example, a binding profile can be determined by contacting a

binding protein with a population of randomized target sequences to identify a sub-population of target sequences bound by that particular binding protein.

A "zinc finger binding protein" is a protein or segment within a larger protein that binds DNA, RNA and/or protein in a sequence-specific manner as a result of stabilization of protein structure through coordination of a zinc ion. The term zinc finger binding protein is often abbreviated as zinc finger protein or ZFP.

A zinc finger "backbone" is the portion of a zinc finger outside the region involved in DNA major groove interactions; *i.e.*, the regions of the zinc finger outside of residues -1 through +6 of the alpha helix. The backbone comprises the beta strands, the connecting region between the second beta strand and the alpha helix, the portion of the alpha helix distal to the first conserved histidine residue, and the inter-finger linker sequence(s). Thus, a plant zinc finger "backbone" refers to sequences derived from one or more plant ZFPs, where these sequences are not naturally involved in DNA major groove interactions.

As used herein, the term "modified plant" zinc finger protein refers to a zinc finger protein comprising plant ZFP sequences organized in a non-plant ZFP structure, for example to eliminate the long stretches of amino acid sequence between zinc fingers found in many naturally-occurring plant ZFPs. Thus, all, most or some of the sequences in the zinc finger regions of a modified plant ZFP may be derived from a plant. Additionally, modified plant ZFPs in these non-plant structures can further include one or more residues or regions (*e.g.*, fingers) of non-plant origin, such as, for example, naturally-occurring fingers or fingers as may be obtained by design or selection, so long as DNA binding capability is maintained.

A "non-canonical" zinc finger protein is a protein not occurring in nature that has been designed and/or selected so as to differ from the canonical binding domain consensus sequence Cys-Cys-His-His (*e.g.*, Cys2-His2). Thus, non-canonical zinc finger proteins comprise a substitution, addition and/or deletion of at least one amino acid, compared to a naturally occurring zinc finger protein. Non-limiting examples of non-canonical zinc fingers include binding domains comprising Cys-Cys-His-Cys (*e.g.*, C3H) sequences and the like. (See, also International Publication entitled "Modified Zinc Finger Proteins" filed even date herewith, Attorney docket No. 8325-0025.40).

A "designed" zinc finger protein is a protein not occurring in nature whose structure and composition results principally from rational criteria. Criteria for rational design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP designs and binding data, for example as described in co-owned PCT WO 00/42219. A "selected" zinc finger protein is a protein not found in nature whose production results primarily from an empirical process such as phage display, two-hybrid systems and/or interaction trap assays. See *e.g.*, US 5,789,538; US 6,007,988; US 6,013,453; WO 95/19431; WO 96/06166; WO 98/54311 and Joung *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:7382-7387. Selection methods also include ribosome display systems (*e.g.*, PCT WO 00/27878) and mRNA-peptide fusion systems (*e.g.*, US Patent No. 6,207,446; PCT WO 00/47775). Amino acid sequences of polypeptides (*e.g.*, zinc fingers) obtained by selection or design are referred to as "adapted" amino acid sequences. Designed and/or selected ZFPs are modified according to the methods and compositions disclosed herein and may also be referred to as "engineered" ZFPs.

The term "naturally-occurring" is used to describe an object that can be found in nature, as distinct from being artificially produced by a human. For example, naturally occurring plant ZFPs are characterized by long spacers of diverse lengths between adjacent zinc finger components.

Nucleic acid or amino acid sequences are "operably linked" (or "operatively linked") when placed into a functional relationship with one another. For instance, a promoter or enhancer is operably linked to a coding sequence if it regulates, or contributes to the modulation of, the transcription of the coding sequence. Operably linked DNA sequences are typically contiguous, and operably linked amino acid sequences are typically contiguous and in the same reading frame. However, since enhancers generally function when separated from the promoter by up to several kilobases or more and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous. Similarly, certain amino acid sequences that are non-contiguous in a primary polypeptide sequence may nonetheless be operably linked due to, for example folding of a polypeptide chain.

With respect to fusion polypeptides, the term "operatively linked" can refer to the fact that each of the components performs the same function in linkage to the other component as

it would if it were not so linked. For example, with respect to a fusion polypeptide in which a modified plant ZFP DNA-binding domain is fused to a functional domain (or functional fragment thereof), the ZFP DNA-binding domain and the functional domain (or functional fragment thereof) are in operative linkage if, in the fusion polypeptide, the modified plant ZFP DNA-binding domain portion is able to bind its target site and/or its binding site, while the functional domain (or functional fragment thereof) is able to modulate (*e.g.*, activate or repress) transcription.

“Specific binding” between, for example, a ZFP and a specific target site means a binding affinity of at least  $1 \times 10^6 \text{ M}^{-1}$ .

A “fusion molecule” is a molecule in which two or more subunit molecules are linked, preferably covalently. The subunit molecules can be the same chemical type of molecule, or can be different chemical types of molecules. Examples of the first type of fusion molecule include, but are not limited to, fusion polypeptides (for example, a fusion between a modified plant ZFP DNA-binding domain and a functional domain) and fusion nucleic acids (for example, a nucleic acid encoding the fusion polypeptide described herein). Examples of the second type of fusion molecule include, but are not limited to, a fusion between a triplex-forming nucleic acid and a polypeptide, and a fusion between a minor groove binder and a nucleic acid.

A “gene,” for the purposes of the present disclosure, includes a DNA region encoding a gene product (see below), as well as all DNA regions that regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions. Further, a promoter can be a normal cellular promoter or, for example, a promoter of an infecting microorganism such as, for example, a bacterium or a virus.

“Gene expression” refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (*e.g.*, mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA)

or a protein produced by translation of an mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

“Gene activation” and “augmentation of gene expression” refer to any process that results in an increase in production of a gene product. A gene product can be either RNA (including, but not limited to, mRNA, rRNA, tRNA, and structural RNA) or protein. Accordingly, gene activation includes those processes that increase transcription of a gene and/or translation of an mRNA. Examples of gene activation processes which increase transcription include, but are not limited to, those which facilitate formation of a transcription initiation complex, those which increase transcription initiation rate, those which increase transcription elongation rate, those which increase processivity of transcription and those which relieve transcriptional repression (by, for example, blocking the binding of a transcriptional repressor). Gene activation can constitute, for example, inhibition of repression as well as stimulation of expression above an existing level. Examples of gene activation processes that increase translation include those that increase translational initiation, those that increase translational elongation and those that increase mRNA stability. In general, gene activation comprises any detectable increase in the production of a gene product, preferably an increase in production of a gene product by about 2-fold, more preferably from about 2- to about 5-fold or any integral value therebetween, more preferably between about 5- and about 10-fold or any integral value therebetween, more preferably between about 10- and about 20-fold or any integral value therebetween, still more preferably between about 20- and about 50-fold or any integral value therebetween, more preferably between about 50- and about 100-fold or any integral value therebetween, more preferably 100-fold or more.

“Gene repression” and “inhibition of gene expression” refer to any process that results in a decrease in production of a gene product. A gene product can be either RNA (including, but not limited to, mRNA, rRNA, tRNA, and structural RNA) or protein. Accordingly, gene repression includes those processes that decrease transcription of a gene and/or translation of an mRNA. Examples of gene repression processes which decrease transcription include, but

are not limited to, those which inhibit formation of a transcription initiation complex, those which decrease transcription initiation rate, those which decrease transcription elongation rate, those which decrease processivity of transcription and those which antagonize transcriptional activation (by, for example, blocking the binding of a transcriptional activator). Gene repression can constitute, for example, prevention of activation as well as inhibition of expression below an existing level. Examples of gene repression processes that decrease translation include those that decrease translational initiation, those that decrease translational elongation and those that decrease mRNA stability. Transcriptional repression includes both reversible and irreversible inactivation of gene transcription. In general, gene repression comprises any detectable decrease in the production of a gene product, preferably a decrease in production of a gene product by about 2-fold, more preferably from about 2- to about 5-fold or any integral value therebetween, more preferably between about 5- and about 10-fold or any integral value therebetween, more preferably between about 10- and about 20-fold or any integral value therebetween, still more preferably between about 20- and about 50-fold or any integral value therebetween, more preferably between about 50- and about 100-fold or any integral value therebetween, more preferably 100-fold or more. Most preferably, gene repression results in complete inhibition of gene expression, such that no gene product is detectable.

The term "modulate" refers to a change in the quantity, degree or extent of a function. For example, the modified plant zinc finger-nucleotide binding polypeptides disclosed herein can modulate the activity of a promoter sequence by binding to a motif within the promoter, thereby inducing, enhancing or suppressing transcription of a gene operatively linked to the promoter sequence. Alternatively, modulation may include inhibition of transcription of a gene wherein the modified zinc finger-nucleotide binding polypeptide binds to the structural gene and blocks DNA dependent RNA polymerase from reading through the gene, thus inhibiting transcription of the gene. The structural gene may be a normal cellular gene or an oncogene, for example. Alternatively, modulation may include inhibition of translation of a transcript. Thus, "modulation" of gene expression includes both gene activation and gene repression.



Modulation can be assayed by determining any parameter that is indirectly or directly affected by the expression of the target gene. Such parameters include, *e.g.*, changes in RNA or protein levels; changes in protein activity; changes in product levels; changes in downstream gene expression; changes in transcription or activity of reporter genes such as, for example, luciferase, CAT, beta-galactosidase, or GFP (see, *e.g.*, Mistili & Spector, (1997) *Nature Biotechnology* 15:961-964); changes in signal transduction; changes in phosphorylation and dephosphorylation; changes in receptor-ligand interactions; changes in concentrations of second messengers such as, for example, cGMP, cAMP, IP<sub>3</sub>, and Ca<sup>2+</sup>; changes in cell growth, changes in chemical composition (*e.g.*, nutritional value), and/or changes in any functional effect of gene expression. Measurements can be made *in vitro*, *in vivo*, and/or *ex vivo*. Such functional effects can be measured by conventional methods, *e.g.*, measurement of RNA or protein levels, measurement of RNA stability, and/or identification of downstream or reporter gene expression. Readout can be by way of, for example, chemiluminescence, fluorescence, colorimetric reactions, antibody binding, inducible markers, ligand binding assays; changes in intracellular second messengers such as cGMP and inositol triphosphate (IP<sub>3</sub>); changes in intracellular calcium levels; cytokine release, and the like.

"Eucaryotic cells" include, but are not limited to, fungal cells (such as yeast), plant cells, animal cells, mammalian cells and human cells. Similarly, "prokaryotic cells" include, but are not limited to, bacteria.

A "regulatory domain" or "functional domain" refers to a protein or a polypeptide sequence that has transcriptional modulation activity, or that is capable of interacting with proteins and/or protein domains that have transcriptional modulation activity. Typically, a functional domain is covalently or non-covalently linked to a ZFP to modulate transcription of a gene of interest. Alternatively, a ZFP can act, in the absence of a functional domain, to modulate transcription. Furthermore, transcription of a gene of interest can be modulated by a ZFP linked to multiple functional domains.

A "functional fragment" of a protein, polypeptide or nucleic acid is a protein, polypeptide or nucleic acid whose sequence is not identical to the full-length protein, polypeptide or nucleic acid, yet retains the same function as the full-length protein,

polypeptide or nucleic acid. A functional fragment can possess more, fewer, or the same number of residues as the corresponding native molecule, and/or can contain one or more amino acid or nucleotide substitutions. Methods for determining the function of a nucleic acid (e.g., coding function, ability to hybridize to another nucleic acid) are well known in the art. Similarly, methods for determining protein function are well known. For example, the DNA-binding function of a polypeptide can be determined, for example, by filter-binding, electrophoretic mobility-shift, or immunoprecipitation assays. See Ausubel *et al.*, *supra*. The ability of a protein to interact with another protein can be determined, for example, by co-immunoprecipitation, two-hybrid assays or complementation, both genetic and biochemical. See, for example, Fields *et al.* (1989) *Nature* 340:245-246; U.S. Patent No. 5,585,245 and PCT WO 98/44350.

A "target site" or "target sequence" is a sequence that is bound by a binding protein such as, for example, a ZFP. Target sequences can be nucleotide sequences (either DNA or RNA) or amino acid sequences. By way of example, a DNA target sequence for a three-finger ZFP is generally either 9 or 10 nucleotides in length, depending upon the presence and/or nature of cross-strand interactions between the ZFP and the target sequence. Target sequences can be found in any DNA or RNA sequence, including regulatory sequences, exons, introns, or any non-coding sequence.

A "target subsite" or "subsite" is the portion of a DNA target site that is bound by a single zinc finger, excluding cross-strand interactions. Thus, in the absence of cross-strand interactions, a subsite is generally three nucleotides in length. In cases in which a cross-strand interaction occurs (e.g., a "D-able subsite," as described for example in co-owned PCT WO 00/42219) a subsite is four nucleotides in length and overlaps with another 3- or 4-nucleotide subsite.

The term "effective amount" includes that amount which results in the desired result, for example, deactivation of a previously activated gene, activation of a previously repressed gene, or inhibition of transcription of a structural gene or translation of RNA.

### Zinc Finger Proteins

Zinc finger proteins are polypeptides that comprise zinc finger components. For example, zinc finger proteins can have one to thirty-seven fingers, commonly having 2, 3, 4, 5 or 6 fingers. Zinc finger DNA-binding proteins are described, for example, in Miller *et al.* (1985) *EMBO J.* 4:1609-1614; Rhodes *et al.* (1993) *Scientific American* Feb.:56-65; and Klug (1999) *J. Mol. Biol.* 293:215-218. A zinc finger protein recognizes and binds to a target site (sometimes referred to as a target sequence or target segment) that represents a relatively small portion of sequence within a target gene. Each component finger of a zinc finger protein binds to a subsite within the target site. The subsite includes a triplet of three contiguous bases on the same strand (sometimes referred to as the target strand). The three bases in the subsite can be individually denoted the 5' base, the mid base, and the 3' base of the triplet, respectively. The subsite may or may not also include a fourth base on the non-target strand that is the complement of the base immediately 3' of the three contiguous bases on the target strand. The base immediately 3' of the three contiguous bases on the target strand is sometimes referred to as the 3' of the 3' base. Alternatively, the four bases of the target strand in a four base subsite can be numbered 4, 3, 2, and 1, respectively, starting from the 5' base.

Zinc finger proteins have been identified in a variety of species. While plant ZFPs are characterized by long spacers between fingers, non-plant ZFPs have much shorter linkers between-finger regions. An exemplary non-plant ZFP is the human transcription factor, Sp-1. As described in detail in WO 00/42219, each of the three zinc fingers in Sp-1 is approximately 30 amino acids in length and is made up of a beta turn (approximately 12 residues in length), and alpha helix (approximately 10-12 residues in length) and short sequence connecting between the beta turn and the alpha helix of approximately 2 residues and an inter-finger linker sequence of 4-5 residues. Exemplary sequences of the zinc fingers of Sp-1 are shown in co-owned WO 00/42219. Also disclosed in WO 00/42219 is an SP-1 consensus sequence, as described by Berg (1992) *Proc. Natl. Acad. Sci. USA* 89:11,109-11,110, which is useful in the design of targeted zinc finger proteins.

Furthermore, in discussing the specificity-determining regions of a zinc finger, amino acid +1 refers to the first amino acid in the alpha-helical portion of the zinc finger. The

portion of a zinc finger that is generally believed to be responsible for its binding specificity lies between -1 and +6. Amino acid ++2 refers to the amino acid at position +2 in a second zinc finger adjacent (in the C-terminal direction) to the zinc finger under consideration. In certain circumstances, a zinc finger binds to its triplet subsite substantially independently of other fingers in the same zinc finger protein. Accordingly, the binding specificity of a zinc finger protein containing multiple fingers is, to a first approximation, the aggregate of the specificities of its component fingers. For example, if a zinc finger protein is formed from first, second and third fingers that individually bind to triplets XXX, YYY, and ZZZ, the binding specificity of the zinc finger protein is 3'-XXX YYY ZZZ-5'.

The relative order of fingers in a zinc finger protein, from N-terminal to C-terminal, determines the relative order of triplets in the target sequence, in the 3' to 5' direction that will be recognized by the fingers. For example, if a zinc finger protein comprises, from N-terminal to C-terminal, first, second and third fingers that individually bind to the triplets 5'-GAC-3', 5'-GTA-3' and 5'-GGC-3', respectively, then the zinc finger protein binds to the target sequence 5'-GGCGTAGAC-3' (SEQ ID NO: 2). If the zinc finger protein comprises the fingers in another order, for example, second finger, first finger, third finger, then the zinc finger protein binds to a target segment comprising a different permutation of triplets, in this example, 5'-GGCGACGTA-3' (SEQ ID NO: 3). See Berg *et al.* (1996) *Science* 271:1081-1086. The numbering convention used above is standard in the field for the region of a zinc finger conferring binding specificity. The amino acid on the N-terminal side of the first invariant His residue is assigned the number +6, and other amino acids, proceeding in an N-terminal direction, are assigned successively decreasing numbers. The alpha helix generally begins at residue +1 and extends to the residue following the second conserved histidine. The entire helix can therefore be of variable length, *e.g.*, between 11 and 13 residues.

#### A. Modified plant ZFPs

A modified plant zinc finger protein is an amino acid sequence, or variant or fragment thereof, which is capable of binding to a target sequence and which comprises sequences derived from plant sources which have been reassembled in a non-plant ZFP structure. Thus,

one or more of the following regions of a modified plant zinc finger are derived from one or more plant sources: the first beta strand, the second beta strand, the alpha helix, and the linker.

It is to be understood that "non-plant" structure refers to any structure that deviates from typical naturally occurring plant ZFPs. One example of a non-plant ZFP scaffold suitable for providing a template for assembling plant-derived sequences is one in which the number of residues between the second histidine of one finger and the first cysteine of the adjacent, C-terminal finger is relatively short. In contrast to typical non-plant ZFPs, plant ZFPs are characterized by long spacers between adjacent fingers. Thus, in certain embodiments, a non-plant structure refers to ZFPs which contain tandem arrays of zinc fingers, *i.e.*, structures in which there are between 5 and 50 amino acids between fingers, more preferably between 5 and 25 amino acids and even more preferably between 5 and 20 amino acids, or any integer therebetween.

Thus, in certain embodiments, the modified plant ZFPs disclosed herein will not contain the sequence QALGGH (SEQ ID NO:105) in the recognition region, which is highly conserved in many plant ZFPs. *See* Takatsuji, (1999) *Plant Mol. Biol.* 39:1073-1078 and references cited therein. Yet another example of a non-plant ZFP structure is one that comprises both canonical C<sub>2</sub>H<sub>2</sub> fingers and non-canonical (*e.g.*, non- C<sub>2</sub>H<sub>2</sub>) fingers. (See, also International Publication entitled "Modified Zinc Finger Proteins" filed even date herewith, Attorney docket No. 8325-0025.40). Other examples of non-plant structures can be readily determined by those of skill in the art in view of the teachings herein. Furthermore, it is to be understood that the modified plant ZFPs described herein may have one or more of these non-plant organization characteristics.

Thus, although the modified plant ZFPs disclosed herein are composed wholly or partly of plant sequences, they have a non-plant structure. The non-plant structure of the modified plant ZFP can be similar to that of any class of non-plant ZFP, for instance the C<sub>2</sub>H<sub>2</sub> canonical class of ZFPs as exemplified by TFIIIA, Zif268 and Sp-1. Furthermore, the modified plant ZFP can comprise sequences from more than one class of ZFP, and selecting particular DNA binding residues and plant backbone residues to achieve the desired effector functions is within the ordinary skill in the art. The Sp-1 sequence used for construction of targeted zinc finger proteins corresponds to amino acids 531 to 624 in the Sp-1 transcription

factor. Thus, models for design of modified plant ZFPs include, but are not limited to, Sp-1 and an Sp-1 consensus sequence, described by Berg (1992) *Proc. Natl. Acad. Sci. USA* 89:11,109-11,110 and by Shi *et al.* (1995) *Chemistry and Biology* 1:83-89. The amino acid sequences of these ZFP frameworks are disclosed in co-owned PCT WO 00/42219. Fungal ZFPs can also be used as models for design and/or as sources of zinc finger sequences for modified plant ZFPs. See, *e.g.*, WO 96/32475. Other suitable ZFPs are known to those of skill in the art and are described herein. The documents cited herein also disclose methods of assessing binding specificity of modified ZFPs.

Optionally, modified plant ZFPs can include one or more residues not present in a naturally occurring plant zinc finger such as can be obtained by, for example, design and/or selection. For example, one or more sequence in the alpha-helical region, particularly residues involved in target-recognition (*e.g.*, amino acids -1, +2, +3 and +6), can be altered with respect to a naturally occurring plant ZFP. Any recognition sequence can be chosen, for example, by selecting residues known to bind to certain target sequences, determined as described herein and in the references cited herein.

Sequences from any ZFP that is used in the methods described herein can be altered by mutagenesis, substitution, insertion and/or deletion of one or more residues so that the non-recognition plant-derived residues do not correspond exactly to the zinc finger from which they are derived. Preferably, at least 75% of the modified plant ZFP residues will correspond to those of the plant sequences, more often 90%, and most preferably greater than 95%.

In general, modified plant ZFPs are produced by a process of analysis of plant sequences, for example those sequences that are publicly available on any number of databases. Three-dimensional modeling can be used, but is not required. Typically, plant sequences are selected for their homology to non-plant ZFPs, for example, by selecting plant ZFPs that most closely resemble the chosen non-plant ZFP scaffold (*e.g.*, a C<sub>3</sub>H structures and/or C<sub>2</sub>H<sub>2</sub> ZFP structure such as Sp-1 or Sp-1 consensus) and binding mode. The plant sequences are then assembled in a non-plant binding mode structure, for instance as three zinc fingers separated by short linkers, as are present in non-plant ZFPs. Thus, the process of obtaining a modified plant ZFP with a predetermined binding specificity can begin by analysis of naturally occurring plant ZFPs.

Once selected plant sequences have been organized and assembled to reflect a non-plant structure, alterations in the recognition residues (*i.e.*, positions -1 to +6 of the alpha helix) can be made so as to confer a desired binding specificity, for example as described in co-owned WO 00/42219; WO 00/41566; as well as U.S. Patents 5,789,538; 6,007,408; 6,013,453; 6,140,081 and 6,140,466; and PCT publications WO 95/19431, WO 98/54311, WO 00/23464; WO 00/27878; WO98/53057; WO98/53058; WO98/53059; and WO98/53060.

In other embodiments, one or more residues, for example recognition residues, can be derived from non-plant sources and inserted into the modified plant ZFP structure. In particular, non-plant sequences that have previously been shown to bind to specific target sequences can be incorporated into the modified plant ZFP to provide the desired binding specificity. Thus, the modified plant ZFPs can include, one or more non-plant derived residues involved in DNA binding where these binding residues have been designed and/or selected to recognize a particular target site, for example as described.

In certain embodiments, modified plant ZFPs, as disclosed herein, contain additional modifications in their zinc fingers, for example, as described in applications of which the benefit is claimed herein. Such additional modifications include, for example, substitution of a zinc-coordinating amino acid residue (*i.e.*, cysteine and/or histidine) with a different amino acid. A modified ZFP of this type can include any number of zinc finger components, and, in one embodiment, contains three zinc fingers. Typically, the C-terminal-most (*e.g.*, third) finger of the ZFP is substituted in one or more zinc-coordinating residues. The other fingers of the protein can be naturally occurring zinc finger components, modified plant components, canonical C<sub>2</sub>H<sub>2</sub> fingers or combinations of these components.

Also included herein are nucleic acids encoding a ZFP comprising at least one modified plant zinc finger as described herein.

#### B. Linkage

Two or more zinc finger proteins can be linked to have a target site specificity that is, to a first approximation, the aggregate of that of the component zinc finger proteins. For example, a first modified plant zinc finger protein having first, second and third component fingers that respectively bind to XXX, YYY and ZZZ can be linked to a second modified

plant zinc finger protein having first, second and third component fingers with binding specificities, AAA, BBB and CCC. The binding specificity of the combined first and second proteins is thus 5'-CCCBBBAAANZZZYYXX-3' (SEQ ID NO:4), where N indicates a short intervening region (typically 0-5 bases of any type). In this situation, the target site can be viewed as comprising two target segments separated by an intervening segment.

Linkage of zinc fingers and zinc finger proteins can be accomplished using any of the following peptide linkers:

TGEKP (SEQ ID NO: 5) Liu *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:5525-5530.

(G<sub>4</sub>S)<sub>n</sub> (SEQ ID NO: 6) Kim *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:1156-1160.

GGRRGGGS (SEQ ID NO: 7)

LRQRDGERP (SEQ ID NO: 8)

LRQKDGGGSRP (SEQ ID NO: 9)

LRQKD(G<sub>3</sub>S)<sub>2</sub>ERP (SEQ ID NO: 10).

Alternatively, flexible linkers can be rationally designed using computer programs capable of modeling both DNA-binding sites and the peptides themselves, or by phage display methods. In a further variation, non-covalent linkage can be achieved by fusing two zinc finger proteins with domains promoting heterodimer formation of the two zinc finger proteins. For example, one zinc finger protein can be fused with *fos* and the other with *jun* (see Barbas *et al.*, WO 95/119431). Alternatively, dimerization interfaces can be obtained by selection. See, for example, Wang *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:9568-9573.

### C. Fusion Molecules

The modified plant zinc finger proteins described herein can also be used in the design of fusion molecules that facilitate regulation of gene expression, particularly in plants. Thus, in certain embodiments, the compositions and methods disclosed herein involve fusions between at least one of the zinc finger proteins described herein (or functional fragments thereof) and one or more functional domains (or functional fragments thereof), or a polynucleotide encoding such a fusion. The presence of such a fusion molecule in a cell allows a functional domain to be brought into proximity with a sequence in a gene that is bound by the zinc finger portion of the fusion molecule. The



transcriptional regulatory function of the functional domain is then able to act on the gene, by, for example, modulating expression of the gene.

In certain embodiments, fusion proteins comprising a modified plant zinc finger DNA-binding domain and a functional domain are used for modulation of endogenous gene expression as described, for example, in co-owned PCT WO 00/41566. Modulation includes repression and activation of gene expression; the nature of the modulation generally depending on the type of functional domain present in the fusion protein. Any polypeptide sequence or domain capable of influencing gene expression (or functional fragment thereof) that can be fused to a DNA-binding domain, is suitable for use.

An exemplary functional domain for fusing with a ZFP DNA-binding domain, to be used for repressing gene expression, is a KRAB repression domain from the human KOX-1 protein (see, e.g., Thiesen et al., *New Biologist* 2, 363-374 (1990); Margolin et al., *Proc. Natl. Acad. Sci. USA* 91, 4509-4513 (1994); Pengue et al., *Nucl. Acids Res.* 22:2908-2914 (1994); Witzgall et al., *Proc. Natl. Acad. Sci. USA* 91, 4514-4518 (1994). Another suitable repression domain is methyl binding domain protein 2B (MBD-2B) (see, also Hendrich et al. (1999) *Mamm Genome* 10:906-912 for description of MBD proteins). Another useful repression domain is that associated with the v-ErbA protein. See, for example, Damm, et al. (1989) *Nature* 339:593-597; Evans (1989) *Int. J. Cancer Suppl.* 4:26-28; Pain et al. (1990) *New Biol.* 2:284-294; Sap et al. (1989) *Nature* 340:242-244; Zenke et al. (1988) *Cell* 52:107-119; and Zenke et al. (1990) *Cell* 61:1035-1049. Additional exemplary repression domains include, but are not limited to, thyroid hormone receptor (TR), SID, MBD1, MBD2, MBD3, MBD4, MBD-like proteins, members of the DNMT family (e.g., DNMT1, DNMT3A, DNMT3B), Rb, MeCP1 and MeCP2. See, for example, Zhang et al. (2000) *Ann Rev Physiol* 62:439-466; Bird et al. (1999) *Cell* 99:451-454; Tyler et al. (1999) *Cell* 99:443-446; Knoepfler et al. (1999) *Cell* 99:447-450; and Robertson et al. (2000) *Nature Genet.* 25:338-342. Additional exemplary repression domains include, but are not limited to, ROM2 and AtHD2A. See, for example, Chern et al. (1996) *Plant Cell* 8:305-321; and Wu et al. (2000) *Plant J.* 22:19-27.

Suitable domains for achieving activation include the HSV VP16 activation domain (see, e.g., Hagmann et al., *J. Virol.* 71, 5952-5962 (1997)) nuclear hormone receptors (see, e.g., Torchia et al., *Curr. Opin. Cell. Biol.* 10:373-383 (1998)); the p65 subunit of nuclear factor kappa B (Bitko & Barik, *J. Virol.* 72:5610-5618 (1998) and Doyle & Hunt, *Neuroreport* 8:2937-2942 (1997)); Liu et al., *Cancer Gene Ther.* 5:3-28 (1998)), or artificial chimeric functional domains such as VP64 (Seifpal et al., *EMBO J.* 11, 4961-4968 (1992)).

Additional exemplary activation domains include, but are not limited to, p300, CBP, PCAF, SRC1, PVALF, and ERF-2. See, for example, Robyr et al. (2000) *Mol. Endocrinol.* 14:329-347; Collingwood et al. (1999) *J. Mol. Endocrinol.* 23:255-275; Leo et al. (2000) *Gene* 245:1-11; Manteuffel-Cymborowska (1999) *Acta Biochim. Pol.* 46:77-89; McKenna et al. (1999) *J. Steroid Biochem. Mol. Biol.* 69:3-12; Malik et al. (2000) *Trends Biochem. Sci.* 25:277-283; and Lemon et al. (1999) *Curr. Opin. Genet. Dev.* 9:499-504. Additional exemplary activation domains include, but are not limited to, OsGAI, HALF-1, C1, AP1, ARF-5, -6, -7, and -8, CPRF1, CPRF4, MYC-RP/GP, and TRAB1. See, for example, Ogawa et al. (2000) *Gene* 245:21-29; Okanami et al. (1996) *Genes Cells* 1:87-99; Goff et al. (1991) *Genes Dev.* 5:298-309; Cho et al. (1999) *Plant Mol. Biol.* 40:419-429; Ulmason et al. (1999) *Proc. Natl. Acad. Sci. USA* 96:5844-5849; Sprenger-Haussels et al. (2000) *Plant J.* 22:1-8; Gong et al. (1999) *Plant Mol. Biol.* 41:33-44; and Hobo et al. (1999) *Proc. Natl. Acad. Sci. USA* 96:15,348-15,353.

Additional functional domains are disclosed, for example, in co-owned WO 00/41566. Further, insulator domains, chromatin remodeling proteins such as ISWI-containing domains and/or methyl binding domain proteins suitable for use in fusion molecules are described, for example, in co-owned International Publications WO 01/83793 and PCT/US01/42377.

In additional embodiments, targeted remodeling of chromatin, as disclosed, for example, in co-owned International Publication WO 01/83793, can be used to generate one or more sites in plant cell chromatin that are accessible to the binding of a functional domain/modified plant ZFP fusion molecule.

Fusion molecules are constructed by methods of cloning and biochemical conjugation that are well known to those of skill in the art. Fusion molecules comprise a modified plant ZFP binding domain and, for example, a transcriptional activation domain, a transcriptional repression domain, a component of a chromatin remodeling complex, an insulator domain or a functional fragment of any of these domains. In certain embodiments, fusion molecules comprise a modified plant zinc finger protein and at least two functional domains (*e.g.*, an insulator domain or a methyl binding protein domain and, additionally, a transcriptional activation or repression domain). Fusion molecules also optionally comprise a nuclear localization signal (such as, for example, that from the SV40 T-antigen or the maize Opaque-2 NLS) and an epitope tag (such as, for example, FLAG or hemagglutinin). Fusion proteins (and nucleic acids encoding them) are designed such that the translational reading frame is preserved among the components of the fusion.

The fusion molecules disclosed herein comprise a modified plant zinc finger binding protein that binds to a target site. In certain embodiments, the target site is present in an accessible region of cellular chromatin. Accessible regions can be determined as described in co-owned International Publications WO 01/83751 and WO 01/83732. If the target site is not present in an accessible region of cellular chromatin, one or more accessible regions can be generated as described in co-owned International Publication WO 01/83793. In additional embodiments, one or more modified plant zinc finger components of a fusion molecule are capable of binding to cellular chromatin regardless of whether its target site is in an accessible region or not. For example, a ZFP as disclosed herein can be capable of binding to linker DNA and/or to nucleosomal DNA. Examples of this type of "pioneer" DNA binding domain are found in certain steroid receptors and in hepatocyte nuclear factor 3 (HNF3). Cordingley *et al.* (1987) *Cell* 48:261-270; Pina *et al.* (1990) *Cell* 60:719-731; and Cirillo *et al.* (1998) *EMBO J.* 17:244-254.

Methods of gene regulation using a functional domain, targeted to a specific sequence by virtue of a fused DNA binding domain, can achieve modulation of gene expression. Genes so modulated can be endogenous genes or exogenous genes.

Modulation of gene expression can be in the form of repression (*e.g.*, repressing expression of exogenous genes, for example, when the target gene resides in a pathological infecting microorganism, or repression of an endogenous gene of the subject, such as an oncogene or a viral receptor, that contributes to a disease state). As described herein, repression of a specific target gene can be achieved by using a fusion molecule comprising a modified plant zinc finger protein and a functional domain.

Alternatively, modulation can be in the form of activation, if activation of a gene (*e.g.*, a tumor suppressor gene or a transgene) can ameliorate a disease state. In this case, a cell is contacted with any of the fusion molecules described herein, wherein the modified zinc finger portion of the fusion molecule is specific for the target gene. The target gene can be an exogenous gene such as, for example, a transgene, or it can be an endogenous cellular gene residing in cellular chromatin. The functional domain (*e.g.*, insulator domain, activation domain, etc.) enables increased and/or sustained expression of the target gene.

For any such applications, the fusion molecule(s) and/or nucleic acids encoding one or more fusion molecules can be formulated with an acceptable carrier, to facilitate introduction into and/or expression in plant cells, as is known to those of skill in the art.

### **Polynucleotide and Polypeptide Delivery**

The compositions described herein can be provided to the target cell *in vitro* or *in vivo*. In addition, the compositions can be provided as polypeptides, polynucleotides or combination thereof.

#### **A. Delivery of Polynucleotides**

In certain embodiments, the compositions are provided as one or more polynucleotides. Further, as noted above, a modified plant zinc finger protein-containing composition can be designed as a fusion between a polypeptide zinc finger and a functional domain that is encoded by a fusion nucleic acid. In both fusion and non-fusion cases, the nucleic acid can be cloned into intermediate vectors for transformation into prokaryotic or eukaryotic (*e.g.*, plant) cells for replication and/or expression.

Intermediate vectors for storage or manipulation of the nucleic acid or production of protein can be prokaryotic vectors, (e.g., plasmids), shuttle vectors, insect vectors, or viral vectors for example. A nucleic acid encoding a modified plant zinc finger protein can also be cloned into an expression vector, for administration to a bacterial cell, fungal cell, protozoal cell, plant cell, or animal cell, preferably a plant cell.

To obtain expression of a cloned nucleic acid, it is typically subcloned into an expression vector that contains a promoter to direct transcription. Suitable bacterial and eukaryotic promoters are well known in the art and described, e.g., in Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*; and Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990). Bacterial expression systems are available in, e.g., *E. coli*, *Bacillus sp.*, and *Salmonella*. Palva *et al.* (1983) *Gene* 22:229-235. Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available, for example, from Invitrogen, Carlsbad, CA and Clontech, Palo Alto, CA.

Plant expression vectors and reporter genes are also generally known in the art. (See, e.g., Gruber *et al.* (1993) in *Methods of Plant Molecular Biology and Biotechnology*, CRC Press.) Such systems include *in vitro* and *in vivo* recombinant DNA techniques, and any other synthetic or natural recombination. (See, e.g., *Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins*, Owen and Pen eds., John Wiley & Sons, 1996; *Transgenic Plants*, Galun and Breiman eds, Imperial College Press, 1997; *Applied Plant Biotechnology*, Chopra, Malik, and Bhat eds., Science Publishers, Inc., 1999.)

The promoter used to direct expression of the nucleic acid of choice depends on the particular application. For example, a strong constitutive promoter is typically used for expression and purification. In contrast, when a protein is to be used *in vivo*, either a constitutive or an inducible promoter is used, depending on the particular use of the protein. In addition, a weak promoter can be used, when low but sustained levels of protein are required. The promoter typically can also include elements that are responsive to transactivation, e.g., hypoxia response elements and small molecule control

systems such as tet-regulated systems and the RU-486 system. *See, e.g., Gossen et al. (1992) Proc. Natl. Acad. Sci USA 89:5547-5551; Oligino et al. (1998) Gene Ther. 5:491-496; Wang et al. (1997) Gene Ther. 4:432-441; Neering et al. (1996) Blood 88:1147-1155; and Rendahl et al. (1998) Nat. Biotechnol. 16:757-761.*

Promoters suitable for use in plant expression systems include, but are not limited to, viral promoters such as the 35S RNA and 19S RNA promoters of cauliflower mosaic virus (CaMV) (Brisson *et al.* (1984) *Nature* 310:511-514, Example 1); the coat protein promoter of TMV (Takamatsu *et al.* (1987) *EMBO J.* 6:307-311); plant promoters such as the small subunit of RUBISCO (Coruzzi *et al.* (1984) *EMBO J.* 3:1671-1680; Broglie *et al.* (1984) *Science* 224:838-843; plant heat shock promoters, *e.g.*, soybean hsp17.5-E or hsp17.3-B (Gurley *et al.* (1986) *Cell. Biol.* 6:559-565) may be used. Other examples of promoters that may be used in expression vectors comprising nucleotides encoding modified plant ZFPs include the promoter for the small subunit of ribulose-1,5-bisphosphate carboxylase; promoters from tumor-inducing plasmids of *Agrobacterium tumefaciens*, such as the RUBISCO nopaline synthase (NOS) and octopine synthase promoters; bacterial T-DNA promoters such as *mas* and *ocs* promoters; or the figwort mosaic virus 35S promoter.

In a preferred embodiment, the modified plant ZFP polynucleotide sequence is under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Example 3). The caulimovirus family has provided a number of exemplary promoters for transgene expression in plants, in particular, the (CaMV) 35S promoter. (*See, e.g., Kay et al. (1987) Science* 236:1299.) Additional promoters from this family such as the figwort mosaic virus promoter, the Commelina yellow mottle virus promoter, and the rice tungro bacilliform virus promoter have been described in the art, and may also be used in the methods and compositions disclosed herein. (*See, e.g., Sanger et al. (1990) Plant Mol. Biol.* 14:433-443; Medberry *et al.* (1992) *Plant Cell* 4:195-192; Yin and Beachy (1995) *Plant J.* 7:969-980.)

The promoters may be modified, if desired, to affect their control characteristics. For example, the CaMV 35S promoter may be ligated to the portion of the RUBISCO gene that represses the expression of RUBISCO in the absence of light, to create a

promoter that is active in leaves, but not in roots. The resulting chimeric promoter may be used as described herein. Constitutive plant promoters such as actin and ubiquitin, having general expression properties known in the art may be used to express modified plant ZFPs. (See, e.g., McElroy *et al.* (1990) *Plant Cell* 2:163-171; Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689.)

Additionally, depending on the desired tissue, expression may be targeted to the endosperm, aleurone layer, embryo (or its parts as scutellum and cotyledons), pericarp, stem, leaves tubers, roots, etc. Examples of known tissue-specific promoters include the tuber-directed class I patatin promoter, the promoters associated with potato tuber ADPGPP genes, the soybean promoter of  $\beta$ -conglycinin (7S protein) which drives seed-directed transcription, and seed-directed promoters from the zein genes of maize endosperm. (See, e.g., Bevan *et al.*, 1986, *Nucleic Acids Res.* 14: 4625-38; Muller *et al.*, 1990, *Mol. Gen. Genet.* 224: 136-46; Bray, 1987, *Planta* 172: 364-370 ; Pedersen *et al.*, 1982, *Cell* 29: 1015-26.) Additional seed-specific promoters include the phaseolin and napin promoters.

In addition to a promoter, an expression vector typically contains a transcription unit or expression cassette that contains additional elements required for the expression of the nucleic acid in host cells, either prokaryotic or eukaryotic. A typical expression cassette thus contains a promoter operably linked, e.g., to the nucleic acid sequence, and signals required, e.g., for efficient polyadenylation of the transcript, transcriptional termination, ribosome binding, and/or translation termination. Additional elements of the cassette may include, e.g., enhancers, and heterologous spliced intronic signals.

The particular expression vector used to transport the genetic information into the cell is selected with regard to the intended use of the resulting ZFP polypeptide, e.g., expression in plants.

In addition, the recombinant constructs may include plant-expressible selectable or screenable marker genes for isolating, identifying or tracking of plant cells transformed by these constructs. Selectable markers include, but are not limited to, genes that confer antibiotic resistances (e.g., resistance to kanamycin or hygromycin) or herbicide resistance (e.g., resistance to sulfonylurea, phosphinothricin, or glyphosate).

Screenable markers include, but are not limited to, the genes encoding beta-glucuronidase (Jefferson (1987) *Plant Molec Biol. Rep* 5:387-405), luciferase (Ow et al. (1986) *Science* 234:856-859), and the B and C1 gene products that regulate anthocyanin pigment production (Goff et al. (1990) *EMBO J* 9:2517-2522).

Other elements that are optionally included in expression vectors also include a replicon that functions in *E. coli* (or in the prokaryotic host, if other than *E. coli*), a selective marker that functions in a prokaryotic host, e.g., a gene encoding antibiotic resistance, to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the vector to allow insertion of recombinant sequences.

Standard transfection methods can be used to produce bacterial, mammalian, yeast, insect, other cell lines or, preferably, plants that express large quantities of modified plant zinc finger proteins, which can be purified, if desired, using standard techniques. See, e.g., Colley et al. (1989) *J. Biol. Chem.* 264:17619-17622; and *Guide to Protein Purification, in Methods in Enzymology*, vol. 182 (Deutscher, ed.) 1990.

Transformation of non-plant eukaryotic cells and prokaryotic cells are performed according to standard techniques. See, e.g., Morrison (1977) *J. Bacteriol.* 132:349-351; Clark-Curtiss et al. (1983) in *Methods in Enzymology* 101:347-362 (Wu et al., eds).

Transformation systems for plants as also known. (See, e.g., Weissbach & Weissbach, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp. 421-463 (1988); Grierson & Corey, *Plant Molecular Biology*, 2d Ed., Blackie, London, Ch. 7-9 (1988).) For example, *Agrobacterium* is often successfully employed to introduce nucleic acids into plants. Such transformation preferably uses binary *Agrobacterium* T-DNA vectors which can be used to transform dicotyledonous plants, monocotyledonous plants and plant cells (Bevan (1984) *Nuc. Acid Res.* 12:8711-8721; Horsch et al. (1985) *Science* 227:1229-1231; Bevan et al. (1982) *Ann. Rev. Genet* 16:357-384; Rogers et al. (1986) *Methods Enzymol.* 118:627-641; Hernalsteen et al. (1984) *EMBO J* 3:3039-3041). In embodiments that utilize the *Agrobacterium* system for transforming plants, the recombinant DNA constructs typically comprise at least the right T-DNA border sequence flanking the DNA sequences to be transformed into the plant



cell. In preferred embodiments, the sequences to be transferred are flanked by the right and left T-DNA border sequences. The design and construction of such T-DNA based transformation vectors are well known to those skilled in the art.

Other gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (see Paszkowski et al. (1984) *EMBO J* 3:2717-2722, Potrykus et al. (1985) *Molec. Gen. Genet.* 199:169-177; Fromm et al. (1985) *Proc. Nat. Acad. Sci. USA* 82:5824-5828; and Shimamoto (1989) *Nature* 338:274-276); electroporation of plant tissues (D'Halluin et al. (1992) *Plant Cell* 4:1495-1505); microinjection, silicon carbide mediated DNA uptake (Kaepler et al. (1990) *Plant Cell Reporter* 9:415-418), microprojectile bombardment (see Klein et al. (1983) *Proc. Nat. Acad. Sci. USA* 85:4305-4309; and Gordon-Kamm et al. (1990) *Plant Cell* 2:603-618); direct gene transfer, *in vitro* protoplast transformation, plant virus-mediated transformation, liposome-mediated transformation, and ballistic particle acceleration (See, e.g., Paszkowski et al. (1984) *EMBO J* 3:2717-2722; U.S. Patent Nos. 4,684,611; 4,407,956; 4,536,475; Crossway et al., (1986) *Biotechniques* 4:320-334; Riggs et al (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606; Hinchey et al. (1988) *Biotechnology* 6:915-921; U.S. Patent No. 4,945,050.)

A wide variety of host cells, plants and plant cell systems can be used, including, but not limited to, those monocotyledonous and dicotyledonous plants, such as crops including grain crops (e.g., wheat, maize, rice, millet, barley), fruit crops (e.g., tomato, apple, pear, strawberry, orange), forage crops (e.g., alfalfa), root vegetable crops (e.g., carrot, potato, sugar beets, yam), leafy vegetable crops (e.g., lettuce, spinach); flowering plants (e.g., petunia, rose, chrysanthemum), conifers and pine trees (e.g., pine fir, spruce); plants used in phytoremediation (e.g., heavy metal accumulating plants); oil crops (e.g., sunflower, rape seed) and plants used for experimental purposes (e.g., *Arabidopsis*).

Modified plant ZFPs and the resulting gene product the ZFP modulates can also be produced from seed by way of seed-based production techniques using, for example, canola, corn, soybeans, rice and barley seed, and the modified plant ZFP, and/or sequences encoding it, can be recovered during seed germination. See, e.g., PCT

Publication Numbers WO 9940210; WO 9916890; WO 9907206; U.S. Patent No.: 5,866,121; and U.S. Patent No.: 5,792,933; and all references cited therein.

#### B. Delivery of Polypeptides

In additional embodiments, modified plant ZFPs or fusion proteins comprising modified plant ZFPs are administered directly to target plant cells. In certain *in vitro* situations, the target cells are cultured in a medium containing a fusion protein comprising one or more functional domains fused to one or more of the modified plant ZFPs described herein. An important factor in the administration of polypeptide compounds in plants is ensuring that the polypeptide has the ability to traverse a cell wall. However, proteins, viruses, toxins, ballistic methods and the like have the ability to translocate polypeptides across a plant cell wall.

For example, "plasmodesmata" is the term given to explain cell-to-cell transport of endogenous and viral proteins and ribonucleoprotein complexes (RNPCs) in plants. Examples of viruses which can be linked to a modified plant zinc finger polypeptide (or fusion containing the same) for facilitating its uptake into plant cells include, tobacco mosaic virus (Oparka et al. (1997) *Plant J.* 12:781-789; rice phloem thioredoxin (Ishiwatari et al. (1998) *Planta* 205:12-22); potato virus X (Cruz et al. (1998) *Plant Cell* 10:495-510) and the like. Other suitable chemical moieties that provide enhanced cellular uptake can also be linked, either covalently or non-covalently, to the ZFPs. Toxin molecules also have the ability to transport polypeptides across cell walls.

Particle-mediated delivery techniques (e.g., ballistic injection) as described above regarding nucleic acids can also be used to introduce polypeptides into a plant cell.

#### **Applications**

The modified plant zinc finger proteins and fusion molecules disclosed herein, and expression vectors encoding these polypeptides, can be used to modulate the expression of, or the action of, any plant endogenous or exogenous gene or gene product. In such applications, modified plant ZFP-containing compositions can be administered directly to a plant, e.g., to facilitate the modulation of gene expression. Preferably, the

modulated gene is endogenous, for example a gene involved in growth, development, morphology, seed or fruit-bearing ability and the like. The gene product itself may be isolated and, accordingly, modulation of endogenous plant genes can be achieved using plant-derived sequences.

Accordingly, expression of any gene in any organism, for example plants or fungi, can be modulated using the methods and compositions disclosed herein, including therapeutically relevant genes, genes of infecting microorganisms, viral genes, and genes whose expression is modulated in the processes of drug discovery and/or target validation. Such genes include, but are not limited to, Wilms' third tumor gene (WT3), vascular endothelial growth factors (VEGFs), VEGF receptors (*e.g.*, *flt* and *flk*) CCR-5, low density lipoprotein receptor (LDLR), estrogen receptor, HER-2/neu, BRCA-1, BRCA-2, phosphoenolpyruvate carboxykinase (PEPCK), CYP7, fibrinogen, apolipoprotein A (ApoA), apolipoprotein B (ApoB), renin, phosphoenolpyruvate carboxykinase (PEPCK), CYP7, fibrinogen, nuclear factor  $\kappa$ B (NF- $\kappa$ B), inhibitor of NF- $\kappa$ B (I- $\kappa$ B), tumor necrosis factors (*e.g.*, TNF- $\alpha$ , TNF- $\beta$ ), interleukin-1 (IL-1), FAS (CD95), FAS ligand (CD95L), atrial natriuretic factor, platelet-derived factor (PDF), amyloid precursor protein (APP), tyrosinase, tyrosine hydroxylase,  $\beta$ -aspartyl hydroxylase, alkaline phosphatase, calpains (*e.g.*, CAPN10) neuronal pentraxin receptor, adriamycin response protein, apolipoprotein E (apoE), leptin, leptin receptor, UCP-1, IL-1, IL-1 receptor, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, IL-15, interleukin receptors, G-CSF, GM-CSF, colony stimulating factor, erythropoietin (EPO), platelet-derived growth factor (PDGF), PDGF receptor, fibroblast growth factor (FGF), FGF receptor, PAF, p16, p19, p53, Rb, p21, *myc*, *myb*, globin, dystrophin, eutrophin, cystic fibrosis transmembrane conductance regulator (CFTR), GDNF, nerve growth factor (NGF), NGF receptor, epidermal growth factor (EGF), EGF receptor, transforming growth factors (*e.g.*, TGF- $\alpha$ , TGF- $\beta$ ), fibroblast growth factor (FGF), interferons (*e.g.*, IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$ ), insulin-related growth factor-1 (IGF-1), angiostatin, ICAM-1, signal transducer and activator of transcription (STAT), androgen receptors, e-cadherin, cathepsins (*e.g.*, cathepsin W), topoisomerase, telomerase, *bcl*, *bcl-2*, *Bax*, T Cell-specific tyrosine kinase (*Lck*), p38 mitogen-activated protein kinase, protein tyrosine phosphatase

(hPTP), adenylate cyclase, guanylate cyclase,  $\alpha 7$  neuronal nicotinic acetylcholine receptor, 5-hydroxytryptamine (serotonin)-2A receptor, transcription elongation factor-3 (TEF-3), phosphatidylcholine transferase, *fz*, PTI-1, polygalacturonase, EPSP synthase, FAD2-1,  $\Delta$ -9 desaturase,  $\Delta$ -12 desaturase,  $\Delta$ -15 desaturase, acetyl-Coenzyme A carboxylase, acyl-ACP thioesterase, ADP-glucose pyrophosphorylase, starch synthase, cellulose synthase, sucrose synthase, fatty acid hydroperoxide lyase, and peroxisome proliferator-activated receptors, such as PPAR- $\gamma 2$ .

Expression of human, mammalian, bacterial, fungal, protozoal, Archaeal, plant and viral genes can be modulated; viral genes include, but are not limited to, hepatitis virus genes such as, for example, HBV-C, HBV-S, HBV-X and HBV-P; and HIV genes such as, for example, *tat* and *rev*. Modulation of expression of genes encoding antigens of a pathogenic organism can be achieved using the disclosed methods and compositions.

In other embodiments, the modulated gene can be exogenous, for example, a transgene that has been inserted into the plant. Techniques for generating transgenic plants are known in the art (see, *e.g.*, Swain W F (1991) *TIBTECH* 9: 107-109; Ma J K C et al. (1994) *Eur J Immunology* 24: 131-138; Hiatt A et al. (1992) *FEBS Letters* 307:71-75; Hein M B et al. (1991) *Biotechnology Progress* 7: 455-461; Duering K (1990) *Plant Molecular Biology* 15: 281-294). As with endogenous genes, the modified plant ZFP (or fusion polypeptides comprising the modified plant ZFPs described herein) can then modulate expression of a transgene, for example to produce a protein product of interest, without the need for regulatory molecules derived primarily from non-plant (*e.g.*, animal) sources.

Accordingly, the compositions and methods disclosed herein can be used to facilitate a number of processes involving transcriptional regulation in plants. These processes include, but are not limited to, transcription, replication, recombination, repair, integration, maintenance of telomeres, processes involved in chromosome stability and disjunction, and maintenance and propagation of chromatin structures. The methods and compositions disclosed herein can be used to affect any of these processes, as well as any other process that can be influenced by ZFPs or ZFP fusions.

Additional exemplary applications for modulation of gene expression in plant cells using modified plant ZFPs include, for example, the optimization of crop traits affecting nutritional value, yield, stress tolerance, pathogen resistance, and resistance to agrochemicals (*e.g.* insecticides and/or herbicides). In addition, targeted gene regulation can be used to study gene function in plants, and to adapt plants for use as biological factories for the production of pharmaceutical compounds or industrial chemicals.

In preferred embodiments, one or more of the molecules described herein are used to achieve targeted activation or repression of gene expression, *e.g.*, based upon the target site specificity of the modified plant ZFP. In another embodiment, one or more of the molecules described herein are used to achieve reactivation of a gene, for example a developmentally silenced gene; or to achieve sustained activation of a transgene. A modified plant ZFP can be targeted to a region outside of the coding region of the gene of interest and, in certain embodiments, is targeted to a region outside of known regulatory region(s) of the gene. In these embodiments, additional molecules, exogenous and/or endogenous, can optionally be used to facilitate repression or activation of gene expression. The additional molecules can also be fusion molecules, for example, fusions between a ZFP and a functional domain such as an activation or repression domain. See, for example, co-owned WO 00/41566.

In other applications, modified plant ZFPs and other DNA- and/or RNA-binding proteins are used in diagnostic methods for sequence-specific detection of target nucleic acid in a sample. For example, modified plant ZFPs can be used to detect variant alleles associated with a phenotype in a plant. As an example, modified plant ZFPs can be used to detect the presence of particular mRNA species or cDNA in a complex mixtures of mRNAs or cDNAs. As a further example, modified plant ZFPs can be used to quantify the copy number of a gene in a sample. A suitable format for performing diagnostic assays employs modified plant ZFPs linked to a domain that allows immobilization of the ZFP on a solid support such as, for example, a microtiter plate or an ELISA plate. The immobilized ZFP is contacted with a sample suspected of containing a target nucleic acid under conditions in which binding between the modified ZFP and its target sequence can occur. Typically, nucleic acids in the sample are labeled (*e.g.*, in the course of PCR amplification). Alternatively, unlabelled

nucleic acids can be detected using a second labeled probe nucleic acid. After washing, bound, labeled nucleic acids are detected. Labeling can be direct (*i.e.*, the probe binds directly to the target nucleic acid) or indirect (*i.e.*, probe binds to one or more molecules which themselves bind to the target). Labels can be, for example, radioactive, fluorescent, chemiluminescent and/or enzymatic.

Modified plant ZFPs, as disclosed herein, can also be used in assays that link phenotype to the expression of particular genes. Current methodologies for determination of gene function rely primarily upon either over-expressing a gene of interest or removing a gene of interest from its natural biological setting, and observing the effects. The phenotypic effects resulting from over-expression or knockout are then interpreted as an indication of the role of the gene in the biological system. Up- or down-regulation of gene expression using one or more modified plant ZFPs obviates the necessity of generating transgenic plants for use in these types of assay.

The following examples are presented as illustrative of, but not limiting, the claimed subject matter.

## EXAMPLES

### **Example 1. Production of modified plant zinc finger binding proteins**

This example describes a strategy to select amino acid sequences for plant zinc finger backbones from among existing plant zinc finger sequences, and subsequent conceptual modification of the selected plant zinc finger amino acid sequences to optimize their DNA binding ability. Oligonucleotides used in the preparation of polynucleotides encoding proteins containing these zinc fingers in tandem array are then described.

#### A. Selection of plant zinc finger backbones

A search was conducted for plant zinc fingers whose backbone sequences (*i.e.*, the portion of the zinc finger outside of the -1 through +6 portion of the recognition helix) resembled that of the SP-1 consensus sequence described by Berg (1992) *Proc. Natl. Acad. Sci. USA* 89:11,109-11,110. The sequences selected included the two conserved cysteine residues, a conserved basic residue (lysine or arginine) located two residues to the C-terminal

side of the second (*i.e.* C-terminal) cysteine, a conserved phenylalanine residue located two residues to the C-terminal side of the basic residue, the two conserved histidine residues, and a conserved arginine residue located two residues to the C-terminal side of the first (*i.e.*, N-terminal) conserved histidine. The amino acid sequences of these selected plant zinc finger backbones (compared to the SP-1 consensus sequence) are shown below, with conserved residues shown in bold and X referring to residues located at positions -1 through +6 in the recognition helix (which will differ among different proteins depending upon the target sequence):

```

SP-1 consensus:  YKCPECGKSFSXXXXXXXXHQRTHTGEKP (SEQ ID NO:11)
F1:              KKKSGHECPICFRVFXXXXXXXXHKRSHTGEKP (SEQ ID NO:12)
F2              YKCTVCGKSFXXXXXXXXHKRLHTGEKP (SEQ ID NO:13)
F3              FSCNYCQRKFXXXXXXXXHVRIH (SEQ ID NO:14)
               -5  -1    5

```

The first finger (F1) was chosen because it contained a basic sequence N-terminal to the finger that is also found adjacent to the first finger of SP-1. The finger denoted F1 is a *Petunia* sequence, the F2 and F3 fingers are *Arabidopsis* sequences.

#### B. Modification of plant zinc finger backbones

Two of the three plant zinc fingers (F1 and F3, above) were modified so that their amino acid sequences more closely resembled the sequence of SP-1, as follows. (Note that the sequence of SP-1 is different from the sequence denoted "SP-1 consensus.") In F3, the Y residue at position -2 was converted to a G, and the sequence QNKK (SEQ ID NO:15) was added to the C-terminus of F3. The QNKK sequence is present C-terminal to the third finger of SP-1, and permits greater flexibility of that finger, compared to fingers 1 and 2, which are flanked by the helix-capping sequence T G E K/R K/P (SEQ ID NO:16). Such flexibility can be beneficial when the third finger is modified to contain a non-C<sub>2</sub>H<sub>2</sub> structure. \*\* Finally, several amino acids were removed from the N-terminus of F1. The resulting zinc finger backbones had the following sequences:

KSKGHECPICFRVFKXXXXXXXXHKRSHTGEKP (SEQ ID NO:17)

YKCTVCGKSFSXXXXXXXXHKRLHTGEKP (SEQ ID NO:18)

FSCNYCQRKFGXXXXXXXXHVRIHQNKK (SEQ ID NO:19)

Amino acid residues denoted by X, present in the recognition portion of these zinc fingers, are designed or selected depending upon the desired target site, according to methods disclosed, for example, in co-owned WO 00/41566 and WO 00/42219, and/or references cited *supra*.

### C. Nucleic acid sequences encoding backbones for modified plant ZFPs

The following polynucleotide sequences are used for design of a three-finger plant ZFP that contains the F1, F2 and F3 backbones described above. Polynucleotides encoding multi-finger ZFPs are designed according to an overlapping oligonucleotide method as described in, for example, co-owned WO 00/41566 and WO 00/42219. Oligonucleotides H1, H2 and H3 (below) comprise sequences corresponding to the reverse complement of the recognition helices of fingers 1-3 respectively; accordingly, nucleotides denoted by N will vary depending upon the desired amino acid sequences of the recognition helices, which, in turn, will depend upon the nucleotide sequence of the target site. Oligonucleotides PB1, PB2 and PB3 encode the beta-sheet portions of the zinc fingers, which are common to all constructs. Codons used frequently in *Arabidopsis* and *E. coli* were selected for use in these oligonucleotides.

H1:

5'-CTC ACC GGT GTG AGA ACG CTT GTG NNN NNN NNN NNN NNN NNN NNN CTT  
GAA AAC ACG GAA-3'  
(SEQ ID NO:20)

H2:

5'-TTC ACC AGT ATG AAG ACG CTT ATG NNN NNN NNN NNN NNN NNN NNN AGA  
AAA AGA CTT ACC-3'  
(SEQ ID NO:21)

H3:

5'-CTT CTT GTT CTG GTG GAT ACG CAC GTG NNN NNN NNN NNN NNN NNN NNN  
ACC GAA CTT ACG CTG-3'  
(SEQ ID NO:22)



PB1:

5'-AAGTCTAAGGGTCACGAGTGCCCAATCTGCTTCCGTGTTTTCAAG-3'  
(SEQ ID NO:23)

PB2:

5'-TCTCACACCGGTGAGAAGCCATACAAGTGCACTGTTTGTGGTAAGTCTTTTTCT-3'  
(SEQ ID NO:24)

PB3:

5'-CTTCATACTGGTGAAAAGCCATTCTCTTGCAACTACTGCCAGCGTAAGTTCGGT-3'  
(SEQ ID NO:25)

Briefly, these six oligonucleotides are annealed and amplified by polymerase chain reaction. The initial amplification product is reamplified using primers that are complementary to the initial amplification product and that also contain 5' extensions containing restriction enzyme recognition sites, to facilitate cloning. The second amplification product is inserted into a vector containing, for example, one or more functional domains, nuclear localization sequences, and/or epitope tags. *See*, for example, co-owned WO 00/41566 and WO 00/42219.

**Example 2: Construction of a polynucleotide encoding a modified plant zinc finger protein for binding to a predetermined target sequence**

A modified plant zinc finger protein was designed to recognize the target sequence 5'-GAGGGGGCG-3' (SEQ ID NO:26). Recognition helix sequences for F1, F2 and F3 were determined, as shown in Table 1, and oligonucleotides corresponding to H1, H2 and H3 above, also including sequences encoding these recognition helices, were used for PCR assembly as described above.

Table 1

Finger	Target	Helix sequence	Nucleotide sequence for PCR assembly
F1	GCG	RSDELTR SEQ ID NO:27	5'CTACCGGTGTGAGAACGCTTGTGACGGGTCAACT CGTCAGAACGCTTGAAAACACGGAA-3' (SEQ ID NO:28)
F2	GGG	RSDHLTR SEQ ID NO:29	5'TTACCAGTATGAAGACGCTTATGACGGGTCAAGT GGTCAGAACGAGAAAAAGACTTACC-3' (SEQ ID NO:30)
F3	GAG	RSDNLTR SEQ ID NO:31	5'CTTCTTGTTCTGGTGGATACGCACGTGACGGGTCA AGTTGTGCAACGACCGAACTTACGCTG-3' (SEQ ID NO:32)

Subsequent to the initial amplification, a secondary amplification was conducted, as described above, using the following primers:

PZF: 5'-CGGGGTACCAGGTAAGTCTAAGGGTCAC (SEQ ID NO:33)

PZR: 5'-GCGCGGATCCACCCTTCTTGGTCTGGTGGATACG (SEQ ID NO:34).

PZF includes a KpnI site (underlined) and overlaps the PB1 sequence (overlap indicated in bold). PZR includes a BamHI (underlined) site and overlaps with H3 (indicated in bold).

The secondary amplification product is digested with Kpn I and Bam HI and inserted into an appropriate vector (e.g., YCF3, whose construction is described below) to construct an expression vector encoding a modified plant ZFP fused to a functional domain, for modulation of gene expression in plant cells.

### **Example 3: Construction of Vectors for Expression of Modified Plant ZFPs**

YCF3 was generated as shown schematically in Figure 1. The starting construct was a plasmid containing a CMV promoter, a SV40 nuclear localization sequence (NLS), a ZFP DNA binding domain, a Herpesvirus VP16 transcriptional activation domain and a FLAG epitope tag (pSB5186-NVF). This construct was digested with SpeI to remove the CMV promoter. The larger fragment was gel-purified and self-ligated to make a plasmid termed GF1. GF1 was then digested with KpnI and HindIII, releasing sequences encoding the ZFP domain, the VP16 activation domain, and the FLAG epitope tag, then the larger fragment was ligated to a KpnI/HindIII fragment containing sequences encoding a ZFP binding domain and a VP16 activation domain, named GF2. This resulted in deletion of sequences encoding the FLAG tag from the construct.

GF2 was digested with BamHI and HindIII, releasing a small fragment encoding the VP16 activation domain, and the larger fragment was purified and ligated to a BamHI/HindIII digested PCR fragment containing the maize C1 activation domain (Goff *et al.* (1990) *EMBO J.* 9:2517-2522) (KpnI and HindIII sites were introduced into the PCR fragment through KpnI and HindIII site-containing primers) to generate NCF1. A PCR fragment containing a Maize Opaque-2 NLS was digested with SpeI/KpnI and ligated to the larger fragment from KpnI/SpeI digested NCF1 to produce YCF2. YCF2 was then digested with MluI and SpeI and the larger fragment was ligated to an MluI and SpeI digested PCR fragment containing

the plant-derived CaMV 35S promoter (MluI and SpeI sites were introduced into the PCR fragment through MluI or SpeI site containing primers) to generate the YCF3 vector.

Sequences encoding modified plant ZFP binding domains can be inserted, as KpnI/BamHI fragments, into KpnI/BamHI-digested YCF3 to generate constructs encoding ZFP-functional domain fusion proteins for modulation of gene expression in plant cells. For example, a series of modified plant ZFP domains, described in Example 4 *infra*, were inserted into KpnI/BamHI-digested YCF3 to generate expression vectors encoding modified plant ZFP-activation domain fusion polypeptides that enhance expression of the *Arabidopsis thaliana* GMT gene.

**Example 4: Modified Plant ZFP Designs for Regulation of an *Arabidopsis thaliana* gamma tocopherol methyltransferase (GMT) Gene**

Modified plant zinc finger proteins were designed to recognize various target sequences in the *Arabidopsis* GMT gene (GenBank Accession Number AAD38271. Table 2 shows the nucleotide sequences of the various GMT target sites, and the amino acid sequences of zinc fingers that recognize the target sites. Sequences encoding these binding domains were prepared as described in Example 1 and inserted into YCF3 as described in Example 3.

Table 2

ZFP #	Target	F1	F2	F3
1	GTGGACGAGT (SEQ ID NO:35)	RSDNLAR (SEQ ID NO:36)	DRSNLTR (SEQ ID NO:37)	RSDALTR (SEQ ID NO:38)
2	CGGGATGGGT (SEQ ID NO:39)	RSDHLAR (SEQ ID NO:40)	TSGNLVR (SEQ ID NO:41)	RSDHLRE (SEQ ID NO:42)
3	TGGTGGGTGT (SEQ ID NO:43)	RSDALTR (SEQ ID NO:44)	RSDHLTT (SEQ ID NO:45)	RSDHLTT (SEQ ID NO:46)
4	GAAGAGGATT (SEQ ID NO:47)	QSSNLAR (SEQ ID NO:48)	RSDNLAR (SEQ ID NO:49)	QSGNLTR (SEQ ID NO:50)
5	GAGGAAGGGG (SEQ ID NO:51)	RSDHLAR (SEQ ID NO:52)	QSGNLAR (SEQ ID NO:53)	RSDNLTR (SEQ ID NO:54)
6	TGGGTAGTC (SEQ ID NO:55)	ERGTLAR (SEQ ID NO:56)	QSGSLTR (SEQ ID NO:57)	RSDHLTT (SEQ ID NO:58)
7	GGGGAAGGG (SEQ ID NO:59)	RSDHLTQ (SEQ ID NO:60)	QSGNLAR (SEQ ID NO:61)	RSDHLR (SEQ ID NO:62)
8	GAAGAGGGTG (SEQ ID NO:63)	QSSHLAR (SEQ ID NO:64)	RSDNLAR (SEQ ID NO:65)	QSGNLAR (SEQ ID NO:66)
9	GAGGAGGATG (SEQ ID NO:67)	QSSNLQR (SEQ ID NO:68)	RSDNALR (SEQ ID NO:69)	RSDNLQR (SEQ ID NO:70)
10	GAGGAGGAGG (SEQ ID NO:71)	RSDNALR (SEQ ID NO:72)	RSDNLAR (SEQ ID NO:73)	RSDNLTR (SEQ ID NO:74)
11	GTGGCGGCTG (SEQ ID NO:75)	QSSDLRR (SEQ ID NO:76)	RSDELQR (SEQ ID NO:77)	RSDALTR (SEQ ID NO:78)
12	TGGGGAGAT (SEQ ID NO:79)	QSSNLAR (SEQ ID NO:80)	QSGHLQR (SEQ ID NO:81)	RSDHLTT (SEQ ID NO:82)
13	GAGGAAGCT (SEQ ID NO:83)	QSSDLRR (SEQ ID NO:84)	QSGNLAR (SEQ ID NO:85)	RSDNLTR (SEQ ID NO:86)
14	GCTTGTGGCT (SEQ ID NO:87)	DRSHLTR (SEQ ID NO:88)	TSGHLTT (SEQ ID NO:89)	QSSDLTR (SEQ ID NO:90)
15	GTAGTGGATG (SEQ ID NO:91)	QSSNLAR (SEQ ID NO:92)	RSDALSR (SEQ ID NO:93)	QSGSLTR (SEQ ID NO:94)
16	GTGTGGGATT (SEQ ID NO:95)	QSSNLAR (SEQ ID NO:96)	RSDHLTT (SEQ ID NO:97)	RSDALTR (SEQ ID NO:98)

**Example 5: Modulation of Expression of an *Arabidopsis thaliana* gamma tocopherol methyltransferase (GMT) Gene**

*Arabidopsis thaliana* protoplasts were prepared and transfected with plasmids encoding modified ZFP-activation domain fusion polypeptides. Preparation of protoplasts and polyethylene glycol-mediated transfection were performed as described. Abel *et al.* (1994) *Plant Journal* 5:421-427. The different plasmids contained the modified plant ZFP binding domains described in Table 2, inserted as KpnI/BamHI fragments into YCF3.

At 18 hours after transfection, RNA was isolated from transfected protoplasts, using an RNA extraction kit from Qiagen (Valencia, CA) according to the manufacturer's instructions. The RNA was then treated with DNase (RNase-free), and analyzed for GMT mRNA content by real-time PCR (TaqMan®). Table 3 shows the sequences of the primers and probe used for TaqMan® analysis. Results for GMT mRNA levels were normalized to levels of 18S rRNA. These normalized results are shown in Figure 2 as fold-activation of GMT mRNA levels, compared to protoplasts transfected with carrier DNA (denoted "No ZFP" in Figure 2). The results indicate that expression of the GMT gene was enhanced in protoplasts that were transfected with plasmids encoding fusions between a transcriptional activation domain and a modified plant ZFP binding domain targeted to the GMT gene.

**Table 3**

	SEQUENCE
GMT forward primer	5'-AATGATCTCGCGGCTGCT-3' (SEQ ID NO:99)
GMT reverse primer	5'-GAATGGCTGATCCAACGCAT-3' (SEQ ID NO:100)
GMT probe	5'-TCACTCGCTCATAAGGCTTCCTTCCAAGT-3' (SEQ ID NO:101)
18S forward primer	5'-TGCAACAAACCCCGACTTATG-3' (SEQ ID NO:102)
18S reverse primer	5'-CCCGCGTCGACCTTTTATC-3' (SEQ ID NO:103)
18S probe	5'-AATAAATGCGTCCCTT-3' (SEQ ID NO:104)

Although the foregoing methods and compositions have been described in detail for purposes of clarity of understanding, certain modifications, as known to those of skill in the art, can be practiced within the scope of the appended claims.

CLAIMS

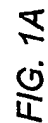
What is claimed is:

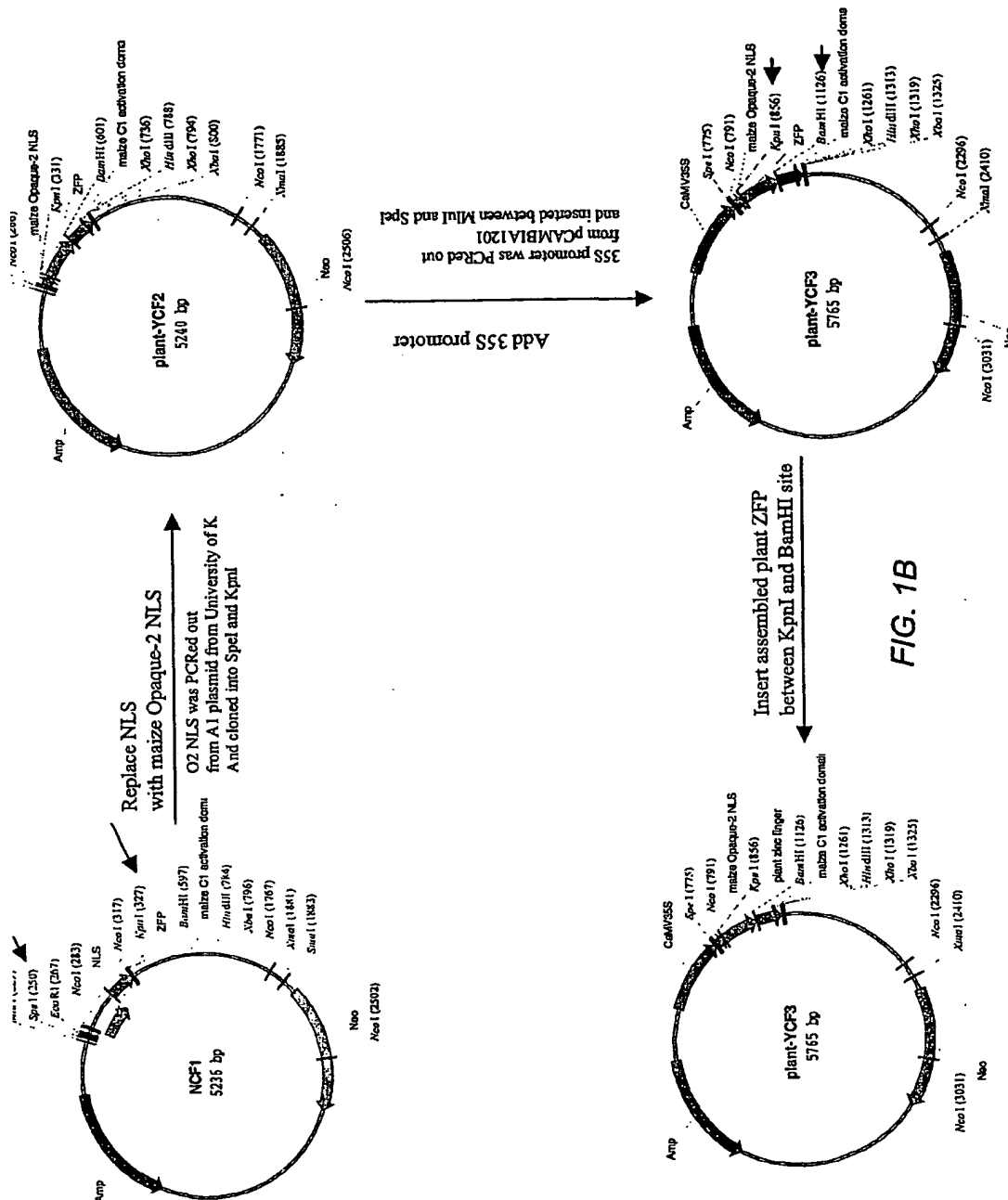
1. A method for modulating gene expression in a plant cell; the method comprising contacting the cell with a modified plant zinc finger protein (ZFP) comprising a tandem array of zinc fingers.
2. The method of claim 1, wherein the protein is expressed in the cell.
3. The method of claim 2, wherein a nucleic acid encoding the protein is introduced into the cell.
4. The method of any of claims 1 to 3, wherein one or more zinc fingers of the modified plant ZFP comprises an adapted amino acid sequence at any one or more of residues -1 through +6 of the recognition helix.
5. The method of claim 4, wherein the adapted amino acid sequence is obtained by rational design.
6. The method of claim 4, wherein the adapted amino acid sequence is obtained by selection.
7. The method of claim 6, wherein selection is accomplished through the use of a method selected from the group consisting of phage display, interaction trap, ribosome display and RNA-peptide fusion.
8. The method of any of claims 1 to 7, wherein the modified plant ZFP comprises a zinc finger backbone derived from two or more different species.

9. The method of any of claims 1 to 7, wherein the modified plant ZFP comprises a zinc finger backbone derived from two or more different plant species.
10. The method of any of claims 1 to 7, wherein the modified plant ZFP comprises zinc finger backbones of fungal origin.
11. The method of any of claims 1 to 10, wherein one or more amino acid residues between one or more of the zinc fingers is deleted.
12. A method for modulating gene expression in a plant cell; the method comprising contacting the cell with a fusion polypeptide comprising (i) a modified plant zinc finger protein (ZFP) comprising a tandem array of zinc fingers and (ii) a functional domain.
13. The method of claim 12, wherein the functional domain is a repressive domain.
14. The method of claim 12, wherein the functional domain is an activation domain.
15. A method for producing a plant having an altered phenotype relative to the wild-type plant comprising the following steps:
  - introducing a nucleic acid encoding a modified plant zinc finger protein (ZFP) comprising a tandem array of zinc fingers into one or more cells of the plant; and
  - expressing the nucleic acid in the plant cell such that the plant has an altered phenotype relative to the wild-type plant.
16. The method of claim 15, wherein the phenotype that is altered is selected from the group consisting of nutritional value, yield, stress tolerance, pathogen resistance, resistance to agrochemicals, production of pharmaceutical compounds, and production of industrial chemicals.

17. A modified plant zinc finger protein comprising one or more zinc fingers that bind to a target site.
18. A fusion polypeptide comprising a modified plan zinc finger protein and at least one functional domain.
19. A polynucleotide encoding the polypeptide of claim 17 or claim 18.
20. An expression vector comprising the polynucleotide of claim 19.
21. A host cell comprising the polynucleotide of claim 19.







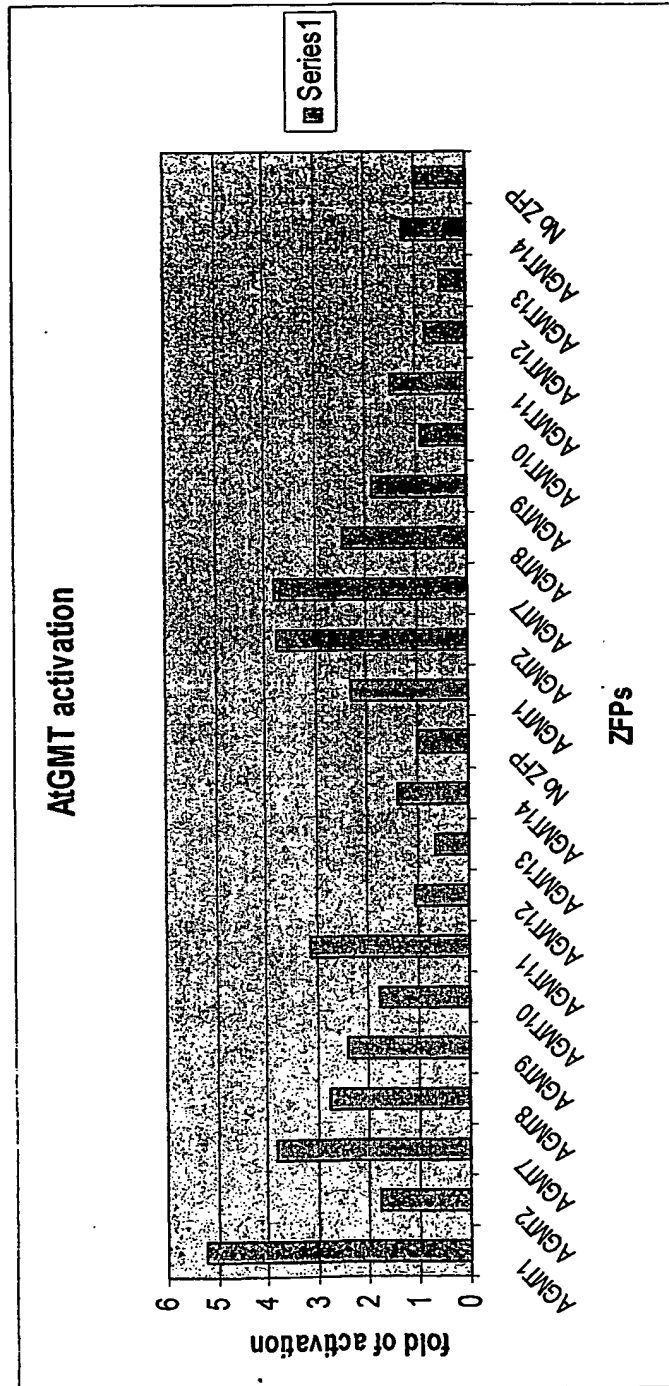


FIG. 2